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Multi-confocal fluorescence correlation spectroscopy in living cells using a CMOS-SPAD array

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Living cells are heterogeneous and rapidly changing biological samples. It is thus desirable to measure molecular concentration and dynamics in many locations at the same time. In this note, we present a multi-confocal setup capable of performing simultaneous Fluorescence Correlation Spectroscopy measurements, by focusing the spots with a Spatial Light Modulator and acquiring data with a monolithic 32×32 single-photon avalanche photodiode array. A post-processing method is proposed to correct cross-talk effects between neighboring spots. We demonstrate the applicability of our system by simultaneously measuring the diffusion of free eGFP molecules at nine different points in living cells.

Fluorescence Correlation Spectroscopy (FCS) is a method designed to assess molecular concentration and dynamics (diffusion, interactions, etc.) by analyzing fluorescence fluctuations originating from a small (generally confocal) observation volume[1]. The relative amplitude of these fluctuations is inversely proportional to the mean number of molecules in the observation volume, while the characteristic time is given by the transit time of the molecules. However, in its standard implementation, the measurement is carried out with only one focused laser spot and lasts a few tens of seconds. This is a severe limitation, since the highly heterogeneous and crowded cellular environment exhibits fast spatial and temporal changes. It is thus important to develop FCS methods that enable simultaneous measurements at different locations within a living cell. One approach is based on two independent spots created by two confocal heads[2]. While offering the same temporal resolution and observation volume as standard FCS, it cannot be easily expanded beyond two spots. On the other hand, spinning disk confocal microscopes make it possible to create numerous spots at a time, with the drawback of observation volumes degraded by aberrations[3,4]. The time resolution can reach $20\mu s$, at the cost of a complicated implementation[5]. We have recently introduced a multi-confocal FCS technique (mFCS) to perform independent and simultaneous dynamics measurements at various locations in living cells[6], using a Spatial Light Modulator (SLM) to create multiple spots and an EMCCD camera that allows a time resolution of $14\mu s$, provided the spots are in a single row.

Newly emerging Complementary Metal Oxide Semiconductor-Single Photon Avalanche Diode (CMOS-SPAD) cameras are very promising for mFCS since they offer the possibility of obtaining 2D diffusion maps without compromise in terms of time resolution. Colyer et al.[7,8] conducted mFCS experiments using an SLM for excitation and a CMOS-SPAD detector for detection in 8 and 64 spots in solutions. More recently, CMOS-SPAD arrays combined with light sheet illumination have been implemented[9,10], but the low signal per molecule of these experiments is not compatible with live cell measurements.

The aim of this work is to investigate the potential of a CMOS-SPAD detector for mFCS measurements in living cells. Compared to our previous work[6] wherein all the spots were aligned, CMOS-SPAD arrays make it possible to arrange spots in 2D patterns, which raises specific issues in terms of crosstalk between closely packed spots. Here, we address these issues and perform live cell mFCS measurements with a time resolution of $2\mu s$.

The CMOS-SPAD detector used in this work is an array of 32×32 SPADs[11] with a pixel pitch of 50μm and an active area of 6.7μm in diameter[12,13]. The readout rate is 500kHz. Approximately 80% of the SPADs feature a dark count rate lower than 50Hz. All measurements were done on such SPADs. The optical setup is built around a commercial inverted microscope (IX70, Olympus). Excitation light at 488 nm is delivered by a CW solid state laser (85-BCD-020, CVI Melles Griot) and spatially filtered by passing through a single-mode fiber. Before reaching the Spatial Light Modulator, the laser beam is expanded so that it covers the largest possible area on the SLM (LCOS-SLM X10468-01, Hamamatsu Photonics) without being clipped. After reflection on the SLM, the beam is coupled onto the microscope objective (Plan-apo $\times$60, NA=1.2, Olympus). The SLM plane is optically conjugated with the back pupil of the objective. The phase map on the SLM is calculated using a spherical wave superposition approach as described previously[6], so that the reflected beamlets are focused in a plane conjugated with the microscope object plane. The collected fluorescence image, which is formed by the microscope tube lens, is reimaged by a pair of lenses onto the CMOS-SPAD detector, resulting in a total optical magnification of $13\times$ from the object plane. This value ensures that the active area of each SPAD is approximately the size of a diffraction-limited fluorescence spot in the image plane, thereby acting as

a confocal pinhole. Our measurement protocol consists of recording the signal detected by each SPAD for 10s. The autocorrelation functions calculated from five consecutive recordings are then averaged and a 3D diffusion model is fitted to the mean to determine the average number of molecules and their diffusion time across the observation volume[14]. By performing calibration FCS measurements in solution (using Rhodamine 6G with a diffusion constant D of $(414 \pm 5)\mu m^2 s^{-1}$ at $T = 25°C$ as a reference value[15]), we have estimated the radial width ω_r of the confocal volume to be 0.241μm which is compatible with the microscope objective diffraction limit. Due to the large pixel pitch of the CMOS-SPAD detector, the minimum distance between spots (if they are placed on adjacent pixels) is $3.8\mu m$ in the sample plane. In the following, we use a pattern of 3×3 spots with this spacing.

For mFCS with a confocal configuration, several closely spaced laser beams are focused into a fluorescent sample, which can be considered infinitely thick (relative to the Rayleigh distance of the focused beam). In this case, the intensity distribution in the image plane created by each beam appears as a bright central spot surrounded by a halo which can be attributed to fluorescence excited out-of-focus. In a 3×3 array, each spot is centered on one SPAD detector, but the latter would still receive contributions from the other beams through their halos which extend to large distances (this effect is named "crosstalk" in the following). These contributions appear as an uncorrelated background that artificially increases the estimated number of molecules in the volume. This problem is more serious for 2D patterns of spots, as compared to 1D arrays[6], since each spot has more neighbors. The consequences can be seen on Figure 1(a) showing the estimated number of molecules in solutions of Dextran-Rhodamine Green solutions with concentrations ranging from 50nM to 700nM in the case of a single spot or an array of 3×3 spots. Since the spots do not have uniform intensities (as can be seen in the inset), they are not affected in the same way by crosstalk: while the number of molecules found in the most intense spot is already significantly increased (green triangles in Figure 1(a)) compared to the single spot case (blue squares), the effect is dramatic for the central spot (red circles) which has more nearest neighbors and a lower intensity. To correct this discrepancy, we propose the following method.

The individual autocorrelation functions from each SPAD m will be corrected by determining the amount of background B_m contained in the total signal S_m by:

$$G_m^{corr}(\tau) - 1 = (G_m^{meas}(\tau) - 1)\left(\frac{S_m}{S_m - B_m}\right)^2 \qquad (1)$$

where $G_m^{corr}(\tau)$ is corrected autocorrelation function and $G_m^{meas}(\tau)$ the measured one[16].

The background B_m includes a constant background mostly due to non-diffracted light reflected by the SLM and a contribution due to crosstalk. The constant background is given by the signals on two "dark" SPADs (located away from the image of the excitation spots) which are saved during each measurement. The Supplemental Material[14] explains how we determined the contribution from crosstalk using a single CCD image of the 33 spots focused into a fluorescent solution. The efficiency of our correction method can be seen in Figure 1(b): after correction, the number of molecules for the central spot (red circles) and the most intense spot (green triangles) of the 3×3 spots array is equivalent to the single spot case (blue squares). Figure 1(c) confirms that the estimated diffusion times are not affected by crosstalk and remain unchanged between single spot and multi-spot cases. Therefore, creating multiple spots does not alter the optical quality of the spots and each of the nine measurement volumes has the same size as the confocal volume for a single spot.

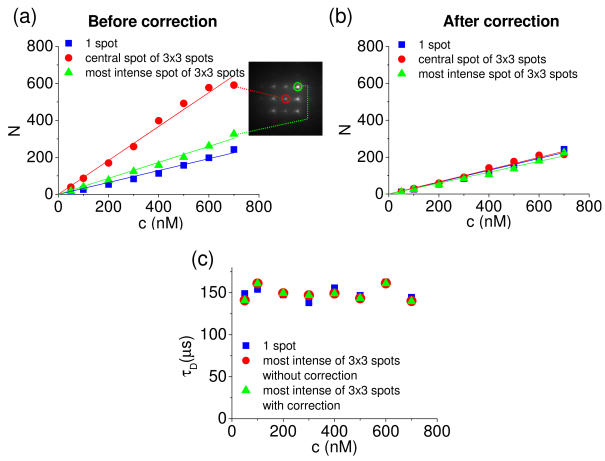

FIG. 1. FCS measurements in solutions of Dextran-Rhodamine Green of various concentrations. The estimated number of molecules (a) for a single spot (blue squares), the central spot (red circles) and the most intense spot (green triangles) of 3×3 spots (shown in inset) have a discrepancy, which disappears (b) after correction. The diffusion time (c) is not affected by crosstalk.

To investigate the potential of our mFCS setup for cellular applications, we have performed measurements in HELA cells expressing the eGFP molecule which is freely diffusing in the nucleus and the cytoplasm. Examples of cells with the 3×3 measurement locations are shown in Figure 2. One may note that, due to the CMOS-SPAD pixel pitch, even when using neighboring pixels, the spacing between spots is such that only a few spots can be placed within a cell. In order to avoid photobleaching, measurements in cells have been performed with a low count-rate per molecule (about 1 kHz). The results presented stem from 11 acquisitions in cells, which have been performed according to the measurement protocol described above. 96 out of the 108 measurement points were located in the nucleus or the cytoplasm. The 96 autocorrelation functions were obtained in a net measurement time of less than 10 minutes which represents a

considerable gain compared to single spot measurements. The measured autocorrelation functions were averaged for both the nuclei and the cytoplasm. Figure 2 shows the results of these measurements. With a lateral width of the observation volume of $0.24\mu m$, we found a diffusion coefficient for eGFP of $D = (21 \pm 3)\mu m^2 s^{-1}$ in the cytoplasm and of $(26 \pm 3)\mu m^2 s^{-1}$ in the nucleus. These two values are similar, which is consistent with the fact that, due to its relatively small size, the diffusional behavior of eGFP is not impacted by the denser and more crowded environment in the nuclei[17].

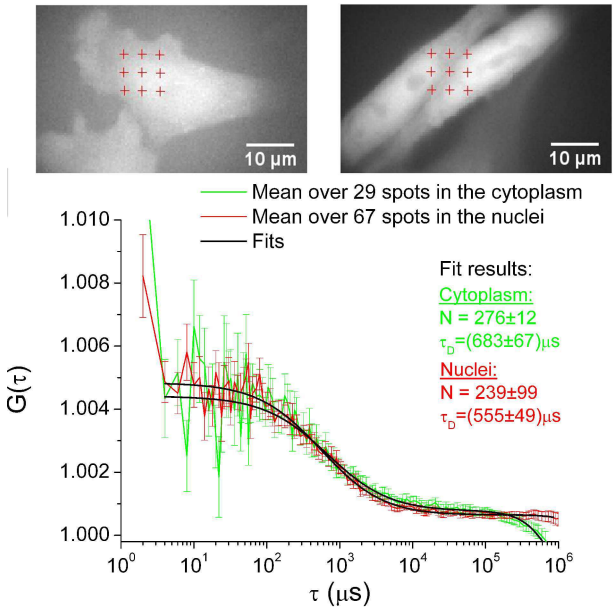

FIG. 2. Upper panel: example of two cells with the location of 3×3 measurement spots. Lower panel: Results of 11 FCS measurements in HELA-eGFP cells; the autocorrelation function in the cytoplasm (in green) is the average of 29 spots, the autocorrelation function in the nuclei (in red) is the average of 67 spots.

These values of the diffusion constant are comparable, albeit lower, to our previous work, where we reported a median value of $37\mu m^2 s^{-1}$ in the nucleus[6]. However, we cannot exclude that some drift of the optical alignment might have slightly biases our estimations.

In this work, we present a scheme for multi-spot parallel FCS measurements using a SLM to generate a 2D matrix of diffraction limited spots and a CMOS-SPAD array for parallel detection. The immediate benefit of this approach is an increased throughput since, in our case, nine parallel measurements have been simultaneously carried out, so that the time needed to obtain the same statistics is significantly reduced.

The CMOS-SPAD array sensor is well-suited for FCS thanks to its single photon sensitivity, low dark count rate and good time resolution ($2\mu s$). Its performances are equivalent to that of the avalanche photodiode, which is the standard detector for single-spot FCS.

When acquiring the signal from an array of spots, a significant background level is present on each spot due to out-of-focus fluorescence excited by neighboring spots and non-diffracted light from the SLM. This problem leads to errors in the estimated number of molecules. We proposed a strategy to evaluate this background for each spot and correct the corresponding autocorrelation function. We showed that the correct number of molecules can be recovered in this way.

Finally, we have demonstrated that our setup is suitable for studying cellular processes by assessing the concentration and diffusion constant of free eGFP in HELA cells. This is the first time, to our knowledge, that parallel FCS measurements using CMOS-SPAD arrays have been carried out on living cells.

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